REMARKS/ARGUMENTS

The Present Invention

The present invention is directed to a method of preparing autologous T-lymphocytes for re-introduction into a patient having cancer, a method of treating a patient having cancer, and related compositions.

The Pending Claims

Claims 1-32 are pending of which claims 15-32 are withdrawn.

The Office Action

The Office Action objects to the abstract as allegedly exceeding the 150-word limit. Claim 4 is rejected under 35 U.S.C. Section 112, second paragraph, as allegedly indefinite. Claims 1 and 14 are rejected under Section 102 (e) as allegedly anticipated by Lupton et al., U.S. Patent 5,874,556 (hereinafter the '556 patent). Claims 1-4 and 14 are rejected under Section 102 (a) as allegedly anticipated by Liu et al., *J. Immunology* 167: 6356-6365 (2001) (hereinafter Liu et al.).

Claims 1-3 and 5-10 are rejected under Section 103 (a) as allegedly unpatentable over the '556 patent in view of Kwak et al., U.S. Patent 6,562,347 (hereinafter the '347 patent). Claims 1-3 are rejected as allegedly unpatentable over the '556 patent in view of Wang et al., *Exp. Opin. Biol. Ther.* 1: 277-290 (2001) (hereinafter Wang et al.). Claims 1 and 11 are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of Einerhand et al., U.S. Patent 6,312,957 (hereinafter the '957 patent). Claims 1, 12, and 13 are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of Roifman et al., *Pediatric Research* 48: 6-11 (2000) (hereinafter Roifman et al.). Claims 1, 12, and 13 are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of Hattori et al., *J. Immunology* 144: 3809-3815 (1990) (hereinafter Hattori et al.). Claims 1, 12, and 13 are rejected as allegedly unpatentable over the '556 patent of Liu et al., each in view of Asami et al., *Eur. J. Haematology* 57: 278-285 (1996) (hereinafter Asami et al.).

Reconsideration of the objection and rejections is hereby requested.

The Amendments to the Specification and Claims

The abstract has been amended to less than 150 words. The specification has been amended to include an Incorporation-by-Reference of Material Submitted Electronically section. Also, the specification has been amended to define the amino acid sequence of the 209-2M peptide, which is incorporated by reference to Liu et al., 167: 6356-6365 (2001). The amino acid sequence of the 209-2M peptide has been added to the Sequence Listing as SEQ ID NO: 5. Claim 4 has been amended to recite that the antigen is the 209-2M peptide (SEQ ID NO: 5). No new matter has been added by way of these amendments.

Discussion of the Indefiniteness Rejection

Claim 4 is rejected as allegedly indefinite for the recitation of "wherein the antigen is amino acids 209-217 of gp100 with a methionine substitution at position 210 (209-2M peptide)," since there allegedly could be multiple differing gp100 amino acid sequences, such that the peptide could have more than one amino acid sequence. The rejection is traversed, because the amino acid sequence of the 209-2M peptide was known at the time of filing the instant application. The 209-2M peptide, as well as its amino acid sequence, is taught by Liu et al, for example. See, the first and third sentences of the second complete paragraph of the left column of page 6357. Also, Kammula et al., *J. Immunol.* 163: 6867-6875 (1999) (a copy of which is attached hereto) teaches the 209-2M peptide along with its amino acid sequence (second sentence of second complete paragraph of left column of page 6868). Therefore, claim 4, including the rejected phrase, is, in fact, clear, such that one of ordinary skill in the art can ascertain the metes and bounds of the claim.

However, in order to advance prosecution and not in acquiescence of the rejection, claim 4 has been amended to define the amino acid sequence eof the 209-2M peptide.

In view of the foregoing, claim 4 is sufficiently clear. Applicants therefore request that the indefiniteness rejection be withdrawn.

Discussion of the Anticipation Rejections

A. The '556 Patent

Claims 1 and 14 are rejected as allegedly anticipated by the '556 patent. Specifically, the Office contends that the '556 patent discloses introducing into CD8+ CTL a retroviral vector expressing IL-2 to reduce the dependency of the cells on T helper cells. The rejection is traversed, because the '556 patent does not disclose each and every element of the claims.

Claim 1 requires obtaining peripheral blood mononuclear cells (PBMCs) from a patient immunized with an antigen of a cancer and stimulating the PBMCs with the antigen of the cancer *in vitro* (see (i) and (ii)). The '556 patent discloses neither (i) nor (ii) as claimed.

Further, claim 1 requires that the retroviral vector does not contain an exogenously introduced gene that enables phenotypic selection (see (iii)(b)), while claim 14 requires that the cells do not contain an exogenously introduced gene that enables phenotypic selection. The '556 patent does not disclose a retroviral vector that lacks an exogenously introduced gene that enables phenotypic selection. In fact, the retroviral vectors disclosed therein comprise the phenotypic selection marker called HyTK, which is a hygromycin phosphotransferase-thymidine kinase fusion gene. Expression of this gene yields a polypeptide that confers hygromycin B resistance for positive selection *in vitro*, and ganciclovir sensitivity for negative selection *in vivo* (column 11, lines12-16).

The Office Action on page 5 asserts that "[t]he retroviral vector as taught by [the '556 patent] does not comprise an exogenously introduced gene that enables phenotypic selection, for example a neo gene or a HSV-TK gene," but does not point to where in the '556 patent this claim limitation is actually disclosed. If the Office Action takes the position that this feature is inherent to the '556 patent, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) In the instant case, such a basis in fact and/or technical reasoning has not been provided. Accordingly, the rejection is improper.

In view of the foregoing, claims 1 and 14 are not anticipated by the '556 patent. Applicants therefore request the withdrawal of the rejection.

B. Liu et al.

Claims 1-4 and 14 are rejected under Section 102 (a) as allegedly anticipated by Liu et al. The Office specifically alleges that Liu et al. discloses the preparation of retroviral vectors containing the IL-2 gene under the control of viral 5' long terminal repeat promoter, which vectors allegedly do not comprise an exogenously introduced gene affecting phenotypic selection. This rejection is traversed, because the retroviral vectors of Liu et al. comprised Yellow Fluorescence Protein (YFP), which is a marker that was used to facilitate the evaluation of transduction efficiency and for positive selection by FACS sorting (see third sentence from the end of the first incomplete paragraph of the left column on page 6357). Accordingly, the retroviral vectors of Liu et al. did, in fact, comprise an exogenously introduced gene affecting phenotypic selection, such that Liu et al. does not teach every element of the rejected claims.

Furthermore, applicant's disclosure of applicant's own work within the year before the application filing date cannot be used against applicant under 35 U.S.C. 102 (a). *In re Katz*, 687 F.2d 450, 215 USPQ 14 (CCPA 1982). Here, Liu et al. represents the work of the two inventors of the instant application, namely, Ke Liu and Steven Rosenberg (see Declaration Under 1.132 of Ke Liu). Moreover, Liu et al. published on December 1, 2001, (see Exhibit A) which was less than one year prior to the filing date of the instant application. Accordingly, Liu et al. is not prior art under 35 U.S.C. 102 (a) to the instant application.

In view of the foregoing, claims 1-4 and 14 are not anticipated by the prior art. Applicants therefore request that the anticipation rejections be withdrawn.

Discussion of the Obviousness Rejections

Claims 1-3 and 5-10 are rejected as allegedly unpatentable over the '556 patent in view of the '347 patent. Claims 1-3 also are rejected as allegedly unpatentable over the '556 patent in view of Wang et al. Claims 1 and 11 are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of the '957 patent. Claims 1, 12, and 13 are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of Roifman et al.

Claims 1, 12, and 13 also are rejected as allegedly unpatentable over the '556 patent or Liu et al., each in view of Hattori et al. Claims 1, 12, and 13 are further rejected as allegedly unpatentable over the '556 patent of Liu et al., each in view of Asami et al. The rejections under Section 103 (a) are traversed for the reasons set forth below.

As a first matter, Liu et al. is not prior art to the instant application. As discussed hereinabove, Liu et al. represents the work of the two inventors, Ke Liu and Steven Rosenberg, and was published less than one year from the filing date of the instant application. As applicant's own work cannot be held against applicant under 35 U.S.C. 102 (a), Liu et al. cannot be used as the basis for an anticipation or obviousness rejection. Accordingly, all rejections under Section 103 based on Liu et al. are improper and should be withdrawn.

The rejections under Section 103 which do not rely on Liu et al. are improper, because the combinations of references do not teach or suggest all of the limitations of the rejected claims. Each of these rejections relies on the '556 patent as the primary reference, which reference allegedly teaches a retroviral vector lacking an exogenously introduced gene that enables phenotypic selection. However, as stated above, the Office merely asserts that the '556 patent teaches this claim limitation without pointing to the teaching(s) in the '556 patent which support the Office's position. Furthermore, contrary to the Office's assertion, the '556 patent, in fact, *does not* teach or suggest such a retroviral vector.

In view of the foregoing, the primary reference of each rejection under Section 103 (the '556 patent) does not teach retroviral vectors which lack the exogenously introduced gene that enables phenotypic selection. None of the secondary references cure the deficiencies of the '556 patent. Accordingly, the rejections under Section 103 cannot stand.

Even if the '556 patent teaches retroviral vectors which lack the exogenously introduced gene that enables phenotypic selection, which it does not, claims 1 to 13 require obtaining PBMCs from patients immunized with a cancer antigen and subsequently stimulating the PBMCs with the cancer antigen *in vitro*. None of the '556 patent and the secondary references teaches obtaining PBMCs from patients immunized with a cancer antigen and subsequently stimulating the PBMCs with the cancer antigen *in vitro*. Therefore, it cannot be said that claims 1-13 are obvious in view of these references.

In view of the foregoing, claims 1-13 are nonobvious in view of the '556 patent in further view of the secondary references cited in the Office Action. Applicants therefore request that the rejections be withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

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Functional Analysis of Antigen-Specific T Lymphocytes by Serial Measurement of Gene Expression in Peripheral Blood Mononuclear Cells and Tumor Specimens

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The cloning of cancer Ags recognized by T cells has provided potentially new tools to enhance immunity against metastatic cancer. The biological monitoring of effective immunization has, however, remained a dilemma. We describe here a sensitive molecular quantitation methodology that allows analysis of in vivo immune response to vaccination. Metastatic melanoma patients were immunized with a synthetically modified peptide epitope (209-2M) from the melanoma self-Ag gp100. Using serial gene expression analysis, we report functional evidence of vaccine-induced CTL reactivity in fresh cells obtained directly from the peripheral blood of postimmunized patients. Further, we demonstrate in vivo localization of vaccine-induced immune response within the tumor microenvironment. The results of these molecular assays provide direct evidence that peptide immunization in humans can result in tumor-specific CTL that localize to metastatic sites. *The Journal of Immunology*, **1999, 163: 6867–6875.**

The existence of systemic and local CTL that recognize tumor Ags provides strong evidence that natural immune response can exist against metastatic cancer in humans. The ease by which melanoma-specific tumor-infiltrating lymphocytes can be isolated from metastatic deposits illustrates this awareness by the immune system (1). However, the presence of melanoma-specific killer cells does not arrest tumor growth. The recent identification and cloning of melanoma-associated Ags recognized by T cells (2, 3) have provided potentially new tools to enhance natural immunity by the same approach that has been successful in the immunization against foreign pathogens. One such self-Ag, gp100, was identified and shown to be a nonmutated differentiation Ag expressed by cells of melanocytic lineage including melanomas, normal melanocytes, and pigmented retinal cells (4). Previously, we reported our experience with the treatment of metastatic melanoma patients with a synthetically modified (to enhance HLA A-2$^+$ binding) gp100 peptide (209-2M)[2] that was emulsified in IFA (5). In vitro sensitization (IVS) and expansion assays of PBMC demonstrated that immunization with the modified peptide was superior to the parental (pa) g209–217 peptide in the induction of CTL reactivity against the 209-pa epitope and HLA-A2$^+$ melanoma cells. Despite measured in vitro PBMC reactivity in 10 of 11 patients who received the 209-2M peptide, no overall clinical responses were observed, although 3 patients exhibited mixed response with complete or partial regression of some lesions. Interestingly, 209-2M immunization combined with the systemic administration of high dose bolus IL-2 resulted in clinical tumor regression in 13 of 31 patients (42%), but only 3 of 19 (16%) patients demonstrated peripheral blood reactivity in the in vitro sensitization assay.

In an attempt to reconcile the dichotomy observed between in vitro generated PBMC reactivity and in vivo tumor regression, we developed a sensitive molecular assay to directly detect specific low precursor CTL reactivity in bulk PBMC and in the local tumor microenvironment. In previous studies involving the immunization of cancer patients, monitoring of immune reactivity has involved single or multiple in vitro restimulation of PBMC to generate cultures with detectable reactivity (5, 6). More direct PBMC analysis with tetrameric HLA/peptide complexes (tHLA) has allowed for measurement of epitope-specific T cell precursor frequency (7) but has been limited by the sensitivity of FACS detection. Further, traditional tetramer analysis does not provide direct assessment of immunological reactivity of the identified cells. The local tumor site, the ultimate target of immunization, has rarely been examined serially in vaccine trials.

In this study, using molecular gene quantitation techniques, we report evidence of specific CTL reactivity in fresh cells obtained directly from the peripheral blood of patients immunized with the 209-2M peptide. We also demonstrate in vivo localization of vaccine-induced immune response by prospectively measuring therapy-specific changes within the tumor microenvironment from sequentially obtained fine needle aspirates of melanoma tumors.

Materials and Methods

Peptides

Each of the peptides utilized in this study was prepared according to Good Manufacturing Practice by Multiple Peptide Systems (San Diego, CA). The identity of each of the peptides was confirmed by mass spectral analysis. Peptide sequences are described below with their applications.

Cultured cell lines

The melanoma cell lines 624.38 Mel (HLA-A2$^+$), 624.28 Mel (HLA-A2$^-$), 888 (A2$^+$) Mel (HLA-A2$^+$), and 888 Mel (HLA-A2$^-$) were established in the Surgery Branch, National Cancer Institute, and cultured as described (8).

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[2] Abbreviations used in this paper: g209-2 M, gp100:209217 (210 M); IVS, in vitro sensitization; pa, parental; FNA, fine needle aspiration; CM, culture medium; sHLA, soluble HLA.

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Clinical protocols

All patients had histologically confirmed metastatic melanoma and had undergone a complete clinical evaluation including measurement and radiographic imaging of all evaluable tumor sites. HLA typing and subtyping for HLA class I was determined on patients' PBL with sequence-specific primer-PCR. All patients provided informed consent before treatment and were verified to have been free of any treatment in the prior month, nor were they receiving immunosuppressive treatment including steroids. Before treatment, patients underwent leukapheresis. PBMC were isolated by Ficoll-Hypaque (ICN, Aurora, OH) separation and were cryopreserved at 10^8 cells/vial and stored at −180°C. In addition, patients underwent fine needle aspiration (FNA) of s.c. metastases immediately before treatment; sampling of the same lesion was repeated after 6–7 wk. Thus, because immunizations were administered at 3-wk intervals, PBMC and FNA were obtained simultaneously before treatment and 3–4 wk after the second vaccination. Aspirate material was examined and verified to contain melanoma cells at the bedside by a cytopathologist. The remainder of the aspirate was placed in ice cold culture medium (CM) consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10 mM HEPES buffer, 100 U/ml penicillin-streptomycin (Biofluids), 0.03% L-glutamine (Biofluids), and 10% heat-inactivated FBS (Biofluids). The sample was immediately brought to the laboratory, centrifuged, placed in RNA lysis buffer (Qiagen, Santa Clarita, CA), and stored at −180°C until RNA isolation was performed.

Two sets of experiments were performed. First, a pilot study was done to assess whether gene measurement techniques could provide information comparable to the assessment of T cell reactivity obtainable with in vitro sensitization experiments as previously described (5). In this first study, vaccination-specific immune reactivity in pre- and postimmunization PBMC was retrospectively compared in 10 patients who had undergone vaccination with a combination of four peptides including gp100, 209-2M (IMQVPFSV); gp100, 280-9V (YLEPGPVTV); MART-1 27–35 (AAGIGILTV), and tyrosinase 368–370D (YMDGTMSQV). Each peptide (1 mg) was administered s.c. emulsified in IFA (Montanide ISA-51, Seppic, France) at 3-wk intervals. Immune reactivity against the 209-pa peptide (ITQVPFSV) (Table I) was analyzed 3–4 wk after the second immunization. Only 209-pa reactivity elicited by vaccination with 209-2M was assessed given that prior in vitro sensitization analysis found no evidence of induction of PBMC reactivity toward the other epitopes.

A second set of experiments was performed after this feasibility study. We evaluated prospectively whether induction of vaccine-specific CTL reactivity could be detected by simultaneous monitoring of tumor and peripheral CTL. With this goal, blood and FNA samples were prospectively collected from patients undergoing simultaneous treatment protocols. Evaluation of response to vaccination was then first tested with the standard in vitro sensitization methods previously described (5, 9). According to results obtained with in vitro sensitization, the patients were divided, before testing with gene expression methods, into three groups defined by the immunological outcome of the treatment received. The first group included patients who received treatment protocols that had shown evidence of effectiveness in inducing enhancement of vaccine-specific CTL reactivity according to in vitro sensitization of pre- and postvaccination PBMC. Nine patients (11 lesions) received "209-2M-based" vaccines: 2 the 209-2M peptide and 7 the modified ES-209-2M peptide (amino-terminal conjugation to adenoviral endoplasmic reticulum signal sequence). Both peptides were emulsified in IFA and administered s.c. at 3-wk intervals as described previously (5). These two treatment categories were combined into one immunological group because previous in vitro sensitization experiments had suggested that the two modalities of g209-2M delivery achieved comparable immunological results with detectable enhancement of g209-pa specific reactivity (data not published). A second group of patients (10 patients/11 lesions), "non-209-2M-based," received immunization with naked DNA encoding the full length gp100 protein with the 209-2M modification (8 patients), the tyrosinase (369–370D) peptide in IFA (1 patient), or adoptive transfer of in vitro generated autologous 209-pa-reactive CTL (1 patient). This group was considered immunologically unresponsive to the treatment, because comparative in vitro sensitization of pre- and postvaccination PBMC had in no case shown evidence of treatment-specific enhancement of T cell reactivity. A final group of patients (8 patients/9 lesions), "IL-2 based," received concomitant IL-2 (Cetus-Oncology Division, Chiron, Emeryville, CA) at a dose of 720,000 IU/kg administered as an i.v. bolus during 15 min starting 1 day after immunization. In this group of patients, immunizations included the ES-209-2M, the gp100 (17–25) (ALLAVGATK), or the tyrosinase (240–244S) DAEKSDICTDEY or (206–214) (AFLPWHRLF) peptides. IL-2 dosing was continued every 8 h until grade 3 or 4 toxicity was reached or to a maximum of 12 doses (10). All peptides were administered s.c. by injecting 1 mg peptide emulsified in IFA. As previously noted (5), the concomitant administration of IL-2 to vaccines nullified the ability to detect vaccine-specific T cell reactivity in posttreatment PBMC. Thus, these patients were grouped in a separate immunological group because they had received a potentially immunogenic treatment (peptide vaccination in IFA), yet no evidence of immunization could be detected in standard in vitro sensitization assays. Although heterogeneous, these treatments were all administered at 3-wk intervals, and all samples were collected immediately before treatment and 3–4 wk after the second vaccination.

Table I. *Quantitative RT-PCR primers and probes*

IFN-γ	(+)-AGCTCTGCATCGTTTTGGGTT[a]
	(−)-GTTCCATTATCCGCTACATCTGAA
	FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA
GM-CSF	(+)-CCCGCCTGGAGCTGTACA
	(−)-AATCTGGGTTGCACAGGAAGTTT
	FAM CCCTTGACCATGATGGCCAGCC-TAMRA
TNF-α	(+)-CCCCAGGGACCTCTCTCTAATC
	(−)-TACAACATGGGCTACAGGCTTG
	FAM-CAGTCAGATCATCTTCTCGAACCCCGAG-TAMRA
IL-2	(+)-ACCAGGATGCTCACATTTAAGTTTT
	(−)-GAGGTTTGAGTTCTTCTTCTAGACACTG
	FAM-CATGCCCAAGAAGGCCACAGAACTG-TAMRA
CD25	(+)-AGCCAGTGGACCAAGCGA
	(−)-TGTGGCTTCATTTTCCCATG
	FAM-TCCAGGTCACTGCAGGGAACCTCC-TAMRA
CD69	(+)-AACAGCTCTTTGCATCCGGA
	(−)-ACCCTTCATGACGTGTTGAGAA
	FAM-CAAGAAAATGATGCCACCAGTCCCCA-TAMRA
CD8	(+)-CCCTGAGCAACTCCATCATGT
	(−)-GTGGGCTTCGCTGGCA
	FAM-TCAGCCACTTCGTGCCGGTCTTC-TAMRA
CD4	(+)-GTCCCTTTTAGGCACTTGCTTCT
	(−)-TCTTTCCCTGAGTGGCTGCT
	FAM-TGCTGCAACTGGCGCTCCTCC-TAMRA
gp100	(+)-GGTTCCTTTTCCGTCACCCT
	(−)-CTCACCGGACGGCACAG
	FAM-ACATTGTCCAGGGTATTGAAAGTGCCGAGAT-TAMRA
β-Actin	(+)-GGCACCCAGCACAATGAAG
	(−)-GCCGATCCACACGGAGTACT
	FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMRA

[a] (+), forward primers; (−), reverse primers; FAM-TAMRA, probe. Concentrations used: 400 nM primers, 150 nM probe.

Epitope-specific T cell staining with HLA-A2 tetramers

PE-tHLA complexes were synthesized as described previously (7). Recombinant HLA-A*0201 heavy chain containing a biotinylation site and recombinant β_2-microglobulin were synthesized and used for refolding of soluble HLA (sHLA) molecules in the presence of a HLA-A*0201 binding peptide. sHLA molecules were prepared for the following epitopes: g209-pa; g209-2M and Flu M1:58–66 (GILGFVFTL). All peptides were commercially synthesized and purified by gel filtration (Princeton Biomolecules, Columbus, OH). The refolding reaction was dialyzed and concentrated for purification of correctly refolded sHLA on gel filtration. Monomeric sHLA was biotinylated with BirA (Avidity, Denver, CO) at the heavy chain and separated from free biotin by gel filtration. Biotinylated sHLA was tetramerized by adding avidin-PE (Pierce, Rockford, IL) at a 4:1 molar ratio. The final concentration of tetramer was adjusted to 2 μg/ml for g209 and g209-2M tHLA and to 1 μg/ml for Flu tHLA. As examined by gel filtration, all tHLA were without detectable free avidin-PE. After overnight depletion of monocytes, nonadherent PBMC were resuspended at 10^6 cells/50 μl ice cold FACS buffer (phosphate buffer plus 5% inactivated FCS, Biofluids), and cells from day 10 CTL cultures were washed and resuspended at 2×10^5 cells/50 μl cold FACS buffer. Cells were incubated

on ice with 1 μg tHLA for 15 min, and incubation was then continued for 30 min with 10 μl anti-CD8 mAb (Becton Dickinson, San Jose, CA). Cells were washed twice in 2 ml cold FACS buffer before analysis by FACS (Becton Dickinson). Two hundred thousand events were acquired. tHLA staining specificity was previously established by extensive analysis of T cell clones specific for each of the described epitopes and by comparative analysis of short term CTL cultures also specific for the above epitopes (11).

In vitro sensitization assessment of peptide-specific CTL reactivity

As previously described (5), cryopreserved PBMC were thawed into CM. Cells were plated at 3×10^6 PBMC in 2 ml medium with 1 μM peptide. IL-2 (300 IU/ml) was added on day 2, and cells were harvested between days 11 and 13 after initiation of the culture. The harvested cells were then stimulated withT2 cells pulsed with 1 μM peptide for 18–24 h at 37°C. IFN-γ release into the supernatant was measured by a standard ELISA assay. Reactivity was scored as positive (+) if IFN-γ release was twice background and >100 pg/ml.

RNA isolation and cDNA synthesis

RNA isolation from PBMCs or fine needle aspirate biopsies was performed in batches containing patient pre- and posttherapy samples with RNeasy mini kits (Qiagen). The RNA was eluted with water and stored at −70°C. For cDNA synthesis, ~1 μg total RNA was transcribed with cDNA transcription reagents (Perkin-Elmer, Foster City, CA) with the use of random hexamers. cDNA was stored at −30°C until quantitative RT-PCR was performed.

Real time quantitative RT-PCR

Measurement of gene expression was performed utilizing the ABI prism 7700 Sequence Detection System (Perkin-Elmer) as previously described (12, 13). Primers and TaqMan probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon-intron junctions to prevent amplification of genomic DNA and to result in amplicons <150 bp to enhance efficiency of PCR amplification. TaqMan probes were labeled at the 5′-end with the reporter dye molecule FAM (6-carboxyfluorescein; emission λ_{max} = 518 nm) and at the 3′-end with the quencher dye molecule TAMARA (6-carboxytetramethylrhodamine; emission λ_{max} = 582 nm). cDNA standards were generated by reverse transcriptase, primer-specific amplification of mRNA of the relevant genes by a technique identical with the one used for the preparation of test cDNA. Amplified cDNA was then purified and quantitated by spectrophotometry (OD_{260}). Copies were calculated using the m.w. of each individual gene amplicon. RT-PCR reactions of cDNA specimens and cDNA standards were conducted in a total volume of 25 μl with 1× *Taq* Man Master Mix (Perkin-Elmer) and primers and probes at optimized concentrations (Table I). Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min. Real time monitoring of fluorescent emission from cleavage of sequence specific probes by the nuclease activity of *taq* polymerase allowed definition of the threshold cycle during the exponential phase of amplification (12). Standard curves were generated for each gene quantitated and were found to have excellent PCR amplification efficiency (90–100%; 100% indicates that in each cycle the amount of template is doubled) as determined by the slope of the standard curves. Linear regression analysis of all standard curves were ≥0.99. Standard curve extrapolation of copy number was performed for the gene of interest as well as an endogenous reference gene for each sample. Normalization of samples was performed by dividing the copies of the gene of interest by copies of the reference gene. All PCR assays were performed in duplicates and reported as the average. A 2-fold difference in gene expression was found to be within the discrimination ability of the assay (data not shown).

Direct molecular assessment of peptide and melanoma-specific CTL reactivity

Cryopreserved PBMC were thawed into CM as described (5). To determine the optimal conditions for assessing direct PBMC reactivity to the immunizing peptides, experiments were conducted using PBMC obtained from patients after immunization. Peptide concentrations ranging from 0.01 to 10 μM were evaluated, as were harvest times ranging from 2 to 24 h, and optimal recovery time of physiological cell metabolism for thawed PBMC. On the basis of these optimization experiments (data not shown), direct PBMC assays were conducted using 3×10^6 PBMC in 2 ml of media, which were allowed to recover by incubation at 37°C in 5% CO_2 for 10 h. Either 1 μM peptide or 1×10^6 melanoma cells were then added to the PBMC and incubated at 37°C in 5% CO_2 for 2 h. No exogenous cytokines or other stimulants were added. The cells were then harvested, and RNA isolation and cDNA transcription were performed. Quantitative RT-PCR was performed for IFN-γ mRNA expression and normalized to copies of CD8 mRNA from the same sample.

Direct molecular assessment of gene expression within the tumor microenvironment

Sequential FNA of the same metastatic tumor site were performed before and after therapy. Aspirate material was examined and verified by a cytopathologist to contain melanoma cells. The remainder of the aspirate was placed in RNA lysis buffer (Qiagen) and stored at −180°C. RNA isolation and cDNA transcriptions from the aspirated material were performed in batches containing patient pre- and posttherapy samples to minimize variability. Quantitative RT-PCR was performed to assess changes in gene expression within the sequential tumor biopsies. Because the aspirates represent only a portion of any tumor and because the immune infiltrate can vary between individual parts of tumors, apparent changes in gene expression between pre- and postvaccination FNA aspirates could only reflect that different areas of the tumor had been probed over time. Thus, the data presented should be interpreted with caution until a larger patient population can be collected and analyzed.

Direct immunofluorescence of fine needle aspirate biopsies

Sequential fine needle aspirates obtained from the same metastatic tumor site were performed before and throughout therapy. Aspirated material was examined and verified by a cytopathologist to contain melanoma cells. Cytospins were performed, and the slides were fixed with acetone for 10 min. tHLA (1 μg) was added to specimens for 2 h at room temperature. Slides were washed vigorously with isotonic saline and visualized with fluorescent microscopy (Olympus, New Hyde Park, NY) at 576 nm.

Statistical analysis

Result reproducibility was tested performing a set of consecutive experiments in which pre- and postvaccination PBMC obtained from the same plasma pheresis from the same patient were independently thawed, stimulated, and processed for cDNA preparation and qRT-PCR. This set of experiments demonstrated that measurements of cytokine mRNA expression were highly reproducible (Table II). Whereas no significant difference could be noted among pre- and postvaccination PBMC that had not received stimulation, a highly significant difference was noted after stimulation of PBMC with 209-pa peptide (unpaired t test, $p < 0.001$). Comparison of pre- vs posttreatment CTL reactivity was assessed by a paired comparison of the fold increment in IFN-γ transcript detection in response to stimulation of a given sample over IFN-γ transcript detection in the same sample that had not been stimulated. To address the accuracy of this method, 24 PBMC samples obtained from HLA-A*0201-expressing patients who had never received vaccination with 209-2M or 209-pa were tested. Furthermore, no evidence of reactivity against either epitope could be detected by in vitro sensitization assays in these samples. The ratio of IFN-γ mRNA transcript detectable in epitope-stimulated PBMC to IFN-γ mRNA transcript detectable in nonstimulated PBMC ranged from 0.6 to1.4 with a median of 0.9 and a mean value of 0.94 ± 0.05 (SEM) at a 95% confidence level of 0.09. To minimize the possibility of falsely considering PBMC immunoreactive, we accepted a 2-fold increase in stimulated-unstimulated IFN-γ transcript ratio (≥2.0 corresponding to >5 SDs above the median) as evidence of epitope-specific reactivity.

Differences in IFN-γ transcript comparing pre- and postvaccination samples were evaluated parametrically by a paired t test. Correlation between gp100 transcript expression in FNA and fold increase in IFN-γ mRNA detection after vaccination was compared by simple linear regression analysis.

Results

Direct molecular assessment of peptide- and melanoma-specific CTL reactivity

To assess direct Ag recognition and reactivity by CTL in peripheral blood, PBMC obtained by leukapheresis from patients before and after two cycles of 209-2M vaccination were directly exposed ex vivo to the 209-pa and 209-2M peptides or melanoma tumor cells. No prior in vitro sensitization or culturing of lymphocytes was performed, nor were exogenous cytokines added to the cells. Because of the low frequency of 209-pa-reactive CTL in bulk PBMC, changes in cytokine release after peptide elicitation were

Table II. *Interassay variability of quantitative RT-PCR measurement of gene expression*[a]

Expt.		No Stimulation	Stimulation with 209-pa (1 μM)
1	PBMC prevaccination	68	76
	PBMC after 2 vaccinations	46	5396
2	PBMC prevaccination	ND	ND
	PBMC after 2 vaccinations	40	5064
3	PBMC prevaccination	83	96
	PBMC after 2 vaccinations	135	6184
4	PBMC prevaccination	65	88
	PBMC after 2 vaccinations	59	5680
Mean ± SEM		72 ± 5.4	87 ± 5.9
		70 ± 22.0	5581 ± 237.1

[a] Data of four consecutive experiments are presented for the patient case presented in Fig. 1*b*. Calculated IFN-γ copies of mRNA standardized over CD8 mRNA copy number after 4 h of PBMC stimulation with g209-pa peptide (1 μM). Each experiment represent a totally independent thaw and stimulation of PBMC with subsequent analysis of IFN-γ mRNA expression. Values represent copies of IFN-γ mRNA per 10^4 copies of CD8 mRNA. 209-pa reactivities twice control (no peptide stimulation) are underlined.

typically below the sensitivity of standard ELISA assays. Therefore, we used real time quantitative RT-PCR to monitor the PBMC for highly specific and quantitative changes in gene expression. As illustrated in Fig. 1*a*, PBMC obtained from a patient after two immunizations with the 209-2M peptide demonstrated a phenotypic increase of $CD8^+$ cells stained with the 209-2M HLA tetramer, as well as the 209-pa HLA tetramer (0.2 and 0.12% of the total PBMC population, respectively). The direct functional analysis of the preimmunized PBMC revealed no detectable reactivity after incubation with either the modified 209-2M or the native 209-pa peptides (Fig. 1*b*). However, the postimmunized PBMC, within a 2-h peptide exposure, demonstrated significant increases in mRNA for the CD69 CTL activation marker, the IL-2α receptor (CD25), and the cytokines IFN-γ, TNF-α, GM-CSF, and IL-2 (Fig. 1*b*). Peptide exposure did not result in changes in the gene expression for IL-1α, IL-1β, IL-4, IL-5, IL-8, IL-12, or IL-15 (data not shown). We consistently noted that postimmunized PBMC demonstrated greater induction of gene expression after exposure to the modified 209-2M peptide when compared with 209-pa peptide (Fig. 1*b*). Physiologically, the cytokine kinetics of IFN-γ, GM-CSF, and IL-2 showed strikingly similar characteristics but the quantitative expression of IFN-γ mRNA was severalfold higher than that of the other two genes. The favorable signal-noise ratio for IFN-γ mRNA expression made this the most suitable single gene to follow as a highly sensitive and specific marker of immune reactivity in our subsequent studies. Reproducible results further delineated that a 2–3 h elicitation time period with 1 μM of peptide to be the optimal parameters for IFN-γ mRNA induction (data not shown). To account for potential variability in the number of $CD8^+$ cells in the samples and reverse transcriptase efficiency during cDNA preparation, normalization of IFN-γ transcripts was performed by dividing by CD8 mRNA copies. CD8 mRNA expression was stable during experiments when compared with traditional housekeeping genes such as β-actin, GAPDH, and rRNA (data not shown). To define the approximate sensitivity limit of this molecular assay, in vitro cultured, 209-pa-reactive CTL clones were spiked into nonreactive autologous PBMC. Significant 209-pa reactivity (compared with response to an irrelevant melanoma Ag epitope, MART 27–35) could be seen at a spiked dilution of 1 CTL clone in 50,000 PBMC (Fig. 1*c*). To determine whether the observed peptide reactivity was associated with tumor reactivity, bulk PBMC were exposed directly to a panel of melanoma cell lines. Response from postimmunized PBMC was found against two HLA-A2+/$gp100^+$ melanomas (624.38 Mel and 888 Mel ($A2^+$)), but not against two HLA-A2-/$gp100^+$ melanomas (624.28 Mel and 888 Mel) as seen in Fig. 1*d*. Our cumulative observations of peripheral lymphocytes demonstrated that 209-2M peptide immunization could result in a significant increase in circulating CTL with highly specific activity directed against a tumor Ag target. Further, these findings were evident in cells, which were obtained directly from patients without any prior in vitro manipulation.

Peptide immunization results in heterogeneous peripheral CTL reactivity

We next examined the relationship between the level of direct 209-pa peptide reactivity in peripheral blood and clinical tumor response. Pre- and postvaccination PBMC were obtained from 10 consecutive patients who were vaccinated with 4 melanoma-associated peptides that included 209-2M (see *Materials and Methods*). PBMC were analyzed for 209-pa reactivity by direct molecular assay and by standard cytokine release assays performed on in vitro sensitized cultures (Table III). In these experiments, evidence of 209-pa immunization for both assays was defined as 209-pa epitope reactivity in postimmunized PBMC that was measured to be twice the background (i.e., no peptide elicitation) and not demonstrable in the preimmunized samples. Reactivity measured by the IVS assay was qualitatively scored as positive (+) or negative (−) because of the inherent in vitro variability of culture growth that could influence quantitative analysis. We found that the IVS assay could generate PBMC cultures with specific antipeptide reactivity (IFN-γ cytokine release) in 7 of 10 patients after immunization, while the direct molecular assay demonstrated immune reactivity (IFN-γ mRNA production) in only 5 of the 10 patients. Patients i and j (Table III) demonstrated qualitative evidence of culture reactivity by the IVS assay but had less than a 2-fold increase by direct molecular assay. Further, for the patients who demonstrated evidence of immunization by both assays (patients *a–e*), direct analysis found significant quantitative heterogeneity among the reactivities, ranging from 2.1- to 76.7-fold over background. Thus, we observed that patients who received identical vaccine therapies mounted very different degrees of peripheral CTL response against the targeted Ag. Despite these findings, there was no correlation between any level of peripheral 209-pa reactivity and objective local tumor response with immunization.

Serial gene expression analysis within the tumor microenvironment demonstrates localization of immune reactivity after 209-2M vaccination

To determine whether lack of tumor regression was due to lack of localized immune response within the tumor, we examined

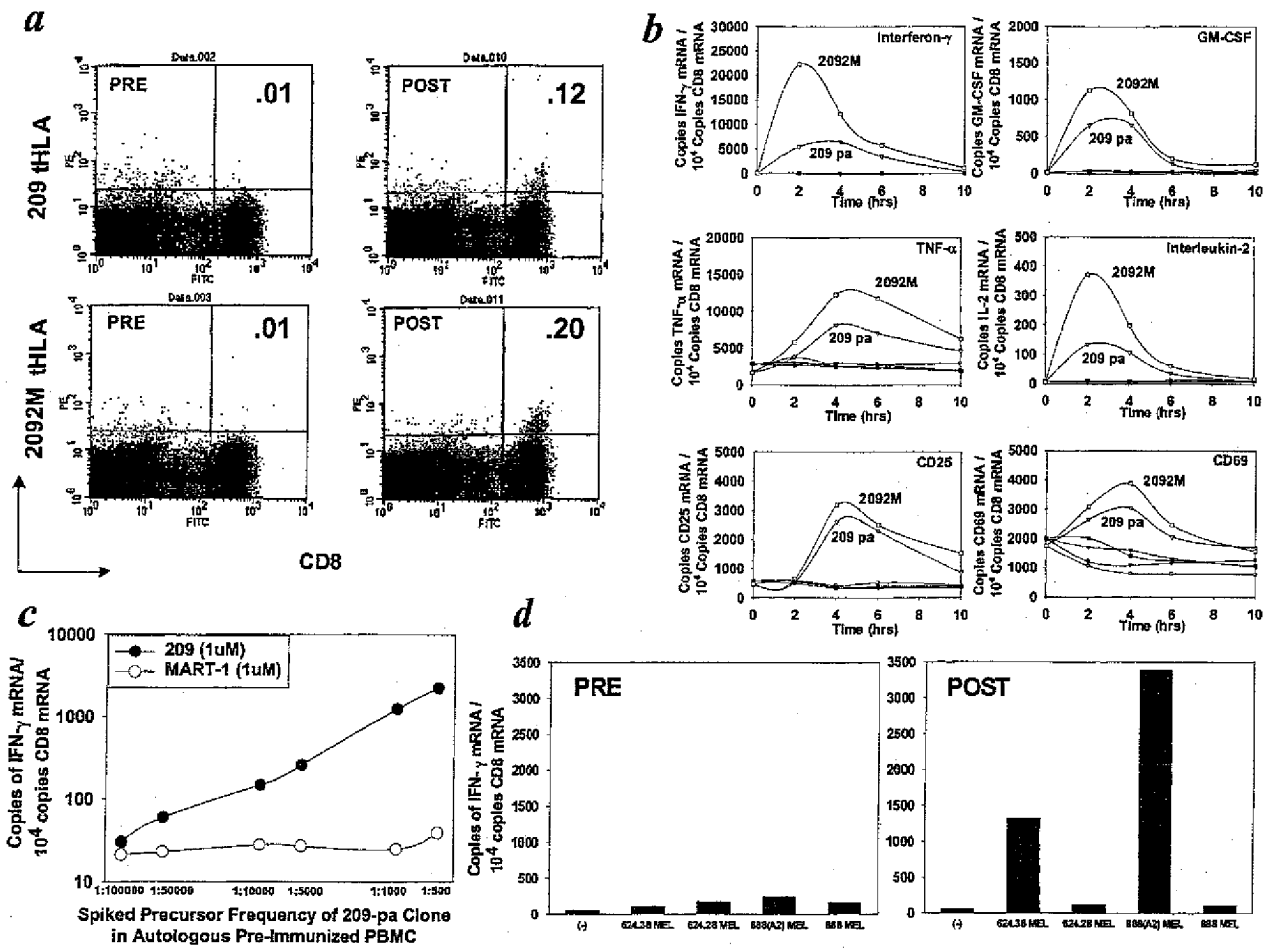

FIGURE 1. Analysis of presence and function of vaccine-specific T cells in PBMC from a highly reactive patient who had received two vaccinations with the 209-2M peptide. *a*, FACS analysis demonstrated the phenotypic increase of 209-pa/209-2M-specific $CD8^+$ cells in the postvaccination PBMC. Numbers in the upper right quadrants (URQ) indicate the percentage of tHLA staining $CD8^+$ T cells calculated according to the formula URQ/(URQ + LRQ) × 100, in which LRQ is lower right quadrant. PBMC populations were gated only according to forward scatter to include cells of size consistent with lymphocytes. Abscissa, staining with anti-CD8 mAb; ordinate, staining with soluble HLA/epitope tetramers (for details about further controls, see *Materials and Methods*). *b*, Kinetics of immune reactivity after direct elicitation of bulk PBMC with 1 μM 209-2M and 209-pa. Real time quantitative RT-PCR performed on PBMC at time points after peptide incubation. Preimmunized PBMC (●), + 209-pa (▼), + 209-2M (■), postimmunized PBMC (○) + 209-pa (▽) + 209-2M (□). *c*, Sensitivity of direct molecular assay; 209-pa-reactive T cell clone was spiked into preimmunized PBMC. Elicitation of 209-pa reactivity (IFN-γ mRNA) could be seen at a spiked dilution of 1 clone in 50,000 PBMC. Negative control was performed with exposure to irrelevant peptide, MART 27–35. *d*, Direct reactivity (IFN-γ mRNA production) of pre- and postimmunized PBMC to HLA-$A2^+$/$gp100^+$ melanomas, 624.38 Mel and 888 Mel ($A2^+$), and HLA-A2-/$gp100^+$ melanomas 624.28 Mel and 888 Mel. These figures represent a complete set of experiments performed in PBMC from the same patient. Each experiment, however, was repeated at least three times on various occasions with similar results with PBMC from the same patient. An exception of Fig. 1*c* were the spiking of the 209-pa-specific clone performed in prevaccination PBMC from other patients because of lack of sufficient material from the same patient. Thus, the results presented in Fig. 1*c* are representative of three experiments with characterized by similar results (range of lowest detectable precursor frequency in the three experiments was 1:100,000 in one experiment and 1:50,000 in the other two experiments.

209-2M therapy-related changes within the local tumor microenvironment of subcutaneous melanoma metastases. Sequential fine needle aspirates of individual lesions in situ were performed on 27 patients before and after vaccine-based immunotherapy. These patients were divided into 3 cohorts based on their treatments (see *Materials and Methods*): a 209-2M cohort (9 patients/11 lesions); and 2 control groups, a non-209-2M cohort (10 patients/11 lesions) and an IL-2 cohort (8 patients/9 lesions). The control therapies represented a variety of experimental vaccine protocols that had shown no evidence of immunization by standard in vitro sensitization assays of peripheral blood. Quantitative RT-PCR was performed from RNA isolated directly from aspirated material obtained from the same lesion before and after treatment. No in vitro culturing or stimulation were performed on the aspirate, so that a true representation of in vivo gene expression and immune reactivity could be ascertained. As demonstrated in Fig. 2*a*, sequential biopsies from the 209-2M cohort showed statistically significant changes in 8 of 11 lesions having a ≥2-fold increase in IFN-γ mRNA expression (normalized to CD8) when pretherapy levels were compared with posttherapy levels for the same lesion (paired *t* test; $p = 0.01$). In contrast, there was no significant change in IFN-γ mRNA expression for the non-209-2M cohort (2 of 11 lesions, $p = 0.40$) or the IL-2 cohort (3 of 9 lesions, $p = 0.19$). Interestingly, direct quantitation of CD8 and CD4 mRNA (normalized to β-actin mRNA), as a representation of cellular immune infiltration in the biopsies, showed no significant change in any of

Table III. *Reactivity of PBMCs from patients immunized with 209-2M peptide in IFA*[a]

Patient	Before Immunization				After Immunization				Clinical Response
	IVS[b]	No pep[c]	209-pa	Fold inc[d]	IVS	No pep	209-pa	Fold inc	
a	(−)	118	101	0.9	(+)	131	715	5.5	NR[e]
b	(−)	316	245	0.8	(+)	142	10,893	76.7	NR
c	(−)	103	107	1.0	(+)	67	546	8.1	NR
d	(−)	38	74	1.9	(+)	50	401	8.0	NR
e	(−)	471	302	0.6	(+)	600	1,259	2.1	NR
f	(−)	73	69	0.9	(−)	110	148	1.3	NR
g	(−)	472	323	0.7	(−)	94	177	1.9	NR
h	(−)	65	52	0.8	(−)	58	70	1.2	NR
i	(−)	47	40	0.9	(+)	59	58	1.0	NR
j	(−)	52	61	1.2	(+)	71	77	1.1	NR

[a] Data refer to the first retrospective pilot study in which IVS results were compared with direct reactivity in PBMC using real time quantitative RT-PCR. Preimmunization samples were obtained 1–3 days before administration of the first vaccination. Postimmunization samples were obtained 3–4 wk after the second vaccination.

[b] PBMCs incubated with 209-2M for 11–13 days before assay against T2 cells alone or pulsed with 209-pa peptide (1 μg/ml). (+), 209-pa reactivity with IFN-γ release (ELISA) that is >100 pg IFN-γ/ml and at least twice that of controls. (−), 209-pa reactivity less than twice that of controls.

[c] Direct quantitative PCR assay. Fresh PBMC were incubated for 2 h with no peptide (No pep) or 209-pa (1 mg/ml). Values represent copies of IFN-γ mRNA per 10^4 copies of CD8 mRNA. 209-pa reactivity twice control (No pep) are underlined.

[d] Fold increase (inc), 209-pa values divided by No pep values. Fold increase ≥2 are underlined.

[e] NR, no response.

the cohorts (Fig. 2, *b* and *c*). However, there was a slight trend toward significance for an increase in CD8 mRNA in the IL-2 cohort ($p = 0.06$).

Although the postimmunized 209-2M cohort showed an overall increase in IFN-γ mRNA expression, the magnitude of this increase was quite variable among the individual lesions, with one

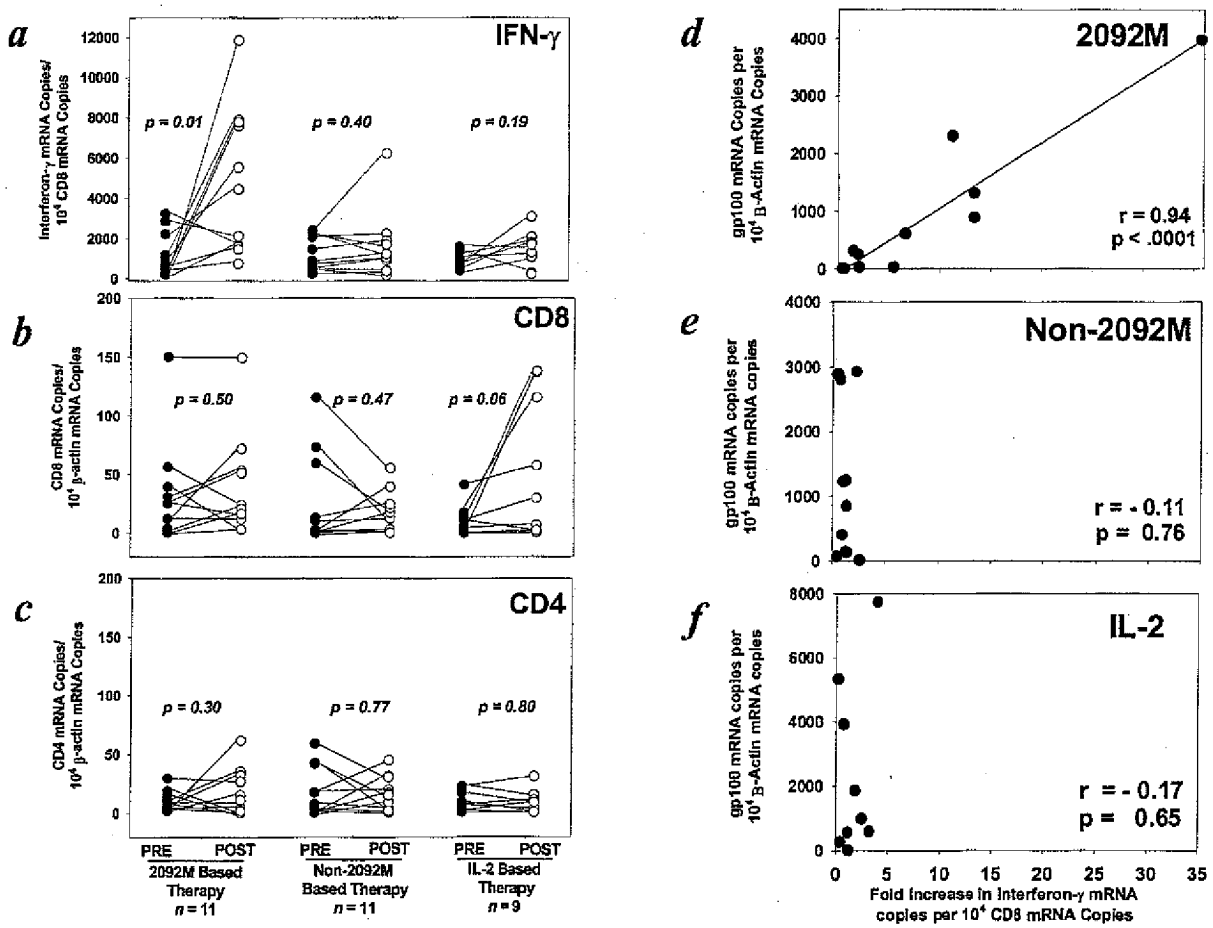

FIGURE 2. Quantitative RT-PCR of sequential final needle aspirates of metastatic melanoma lesions. *a*, IFN-γ mRNA (normalized to CD8 mRNA); *b*, CD8 mRNA; *c*, CD4 mRNA (normalized to β-actin mRNA). Statistical analysis, paired *t* test. Correlation of fold increase in IFN-γ vs gp100 mRNA expression for lesions from 209-2M cohort (*d*), non-209-2M cohort (*e*), and IL-2 cohort (*f*). Correlation between gp100 transcript expression in FNA and fold increase in IFN-γ mRNA detection after vaccination were compared by simple linear regression analysis.

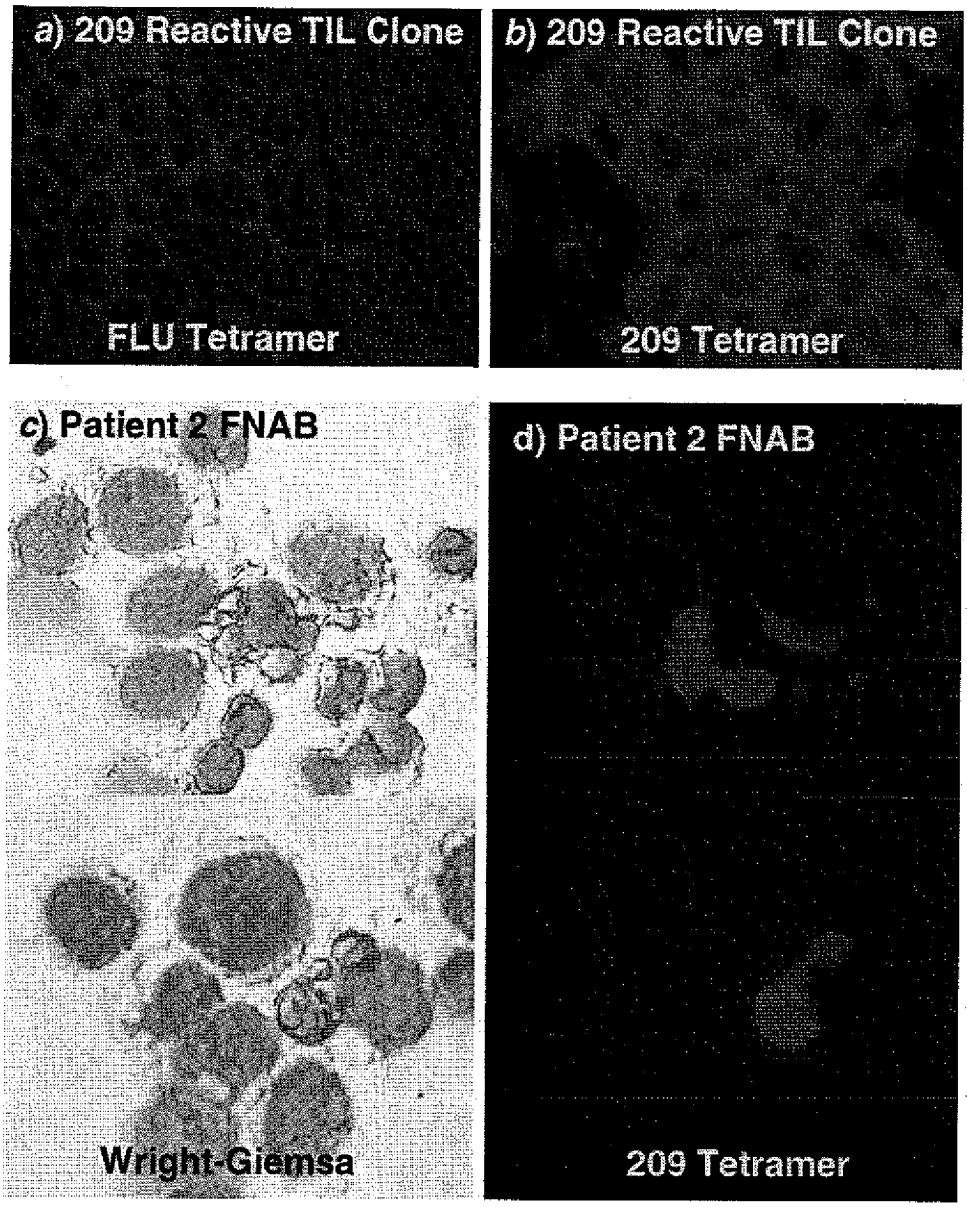

FIGURE 3. Direct immunofluorescence of CTL using 209-pa tHLA. Control 209-pa reactive tumor-infiltrating lymphocyte (TIL) clones stained with Flu tHLA (background staining) (*a*) and 209-pa tHLA (*b*). Fine needle aspirate of metastasis from patient 1 after 2 cycles of 209-2M immunization; staining with Wright-Giemsa (demonstrating melanoma cells and lymphocytes) (*c*) and 209-pa tHLA (fluorescent orange staining of lymphocytes) (*d*). Control staining included tHLA staining of prevaccination fine needle aspiration (FNA) that did not demonstrate evidence of 209-pa specific T cells at tumor site (not shown). Flu tHLA staining was not performed in this FNA because of lack of sufficient material. Previous studies in other FNA have shown that Flu-specific T cells can be identified in material obtained from melanoma metastases probably in relation to blood contamination; however, they are extremely rare and their frequency does not significantly change in relation to vaccination.

lesion showing a 35-fold increase, whereas others showed marginal or no change. In an effort to explain this heterogeneity, we examined the concurrent mRNA expression for the targeted tumor Ag, gp100, in each of the biopsies. As demonstrated in Fig. 2*d*, quantitative RT-PCR for gp100 mRNA expression (normalized to β-actin mRNA) also demonstrated marked heterogeneity, which correlated very highly with the observed tumor changes in IFN-γ mRNA for the 209-2M cohort ($r = 0.94$, $p < 0.0001$). This correlation was not seen in the other two treatment cohorts, whose lesions were obtained and processed in an identical manner and which showed similar variability of gp100 Ag expression (Fig. 2, *e* and *f*). These findings demonstrated that 209-2M immunization (in contrast to other therapies) could result in measurable immunological changes within tumors if appropriate levels of Ag were present. Change in IFN-γ mRNA was used as a parameter rather than the absolute levels of IFN-γ transcript to better represent the impact of therapy and to account for in vivo baseline variability among lesions.

Direct immunofluorescence of fine needle aspirate biopsies demonstrates localization of 209-pa reactive CTL

To visualize the cellular response within the tumor microenvironment after 2092-M vaccination, we examined cytospins prepared from sequential fine needle aspirates of the lesion which showed the greatest change in IFN-γ mRNA levels. As shown in Fig. 3*c*,

Table IV. *Comparison of peripheral vs tumor site monitoring after 209-2M-based peptide vaccination*[a]

Patient	Therapy	Peripheral Monitoring (PBMC)				Tumor Monitoring (Tumor Aspirates)				Tumor Response Size (cm)
		IVS	No pep	209-pa	Fold inc	Tumor site	No pep[b]	Fold inc[c]	gp100[d]	
1	Pre	(−)	54	64	1.2	Right axilla	568		1,242	4
	Post	(+)	47	103	2.2		7,586	13.4	1,310	9
2	Pre	(−)	39	45	1.2	Left thigh	331		1,956	1
	Post	(−)	41	43	1.0		11,865	35.8	3,955	1
3	Pre	(−)	22	24	1.1	Right axilla	1,187		6,186	9
	Post	(−)	30	45	1.5		7,891	6.6	611	25
4	Pre	(−)	52	28	0.5	Left thigh	579		2,865	6
	Post	(−)	35	37	1.1		7,788	13.5	894	20.25
5	Pre	(−)	104	92	0.9	Right thigh	2,231		1,226	1
	Post	(+)	50	72	1.4		4,452	2.0	235	4
6	Pre	(−)	95	84	0.9	Left chest	1,013		1	2
	Post	(+)	42	151	3.6		5,532	5.5	18	5
7	Pre	(−)	59	44	0.7	#1 left medial knee	3,247		0	2.25
	Post	(−)	29	37	1.3		1,649	0.5	1	2.5
						#2 left lateral knee	2,865		1	6.25
							2,131	0.7	1	6.25
8	Pre	(−)	73	69	0.9	#1 right lower thigh	140		4,786	6
	Post	(−)	110	148	1.3		1,585	11.3	2,291	4
						#2 right upper thigh	466		532	16
							759	1.6	312	16
9	Pre	(−)	103	107	1.0	Right groin	692		60	4
	Post	(+)	67	546	8.1		1,474	2.1	12	6

[a] Preimmunization PBMC samples were obtained 1–3 days before administration of the first vaccination. Postimmunization PBMC were obtained 3–4 wk after the second vaccination. FNA samples were obtained within 2 days of the collection of the relative PBMC samples.

[b] Tumor aspirates underwent no peptide exposure (No pep). Quantitative PCR performed directly on fresh sample. Values represent copies of IFN-γ mRNA per 10^4 copies of CD8 mRNA. Post values twice pre values are underlined.

[c] Fold increase (Fold inc) for tumor monitoring = Post values divided by Pre values. Fold increases ≥2 are underlined.

[d] Values represent copies of gp100 mRNA per 10^4 copies of α-actin mRNA.

Wright-Giemsa staining of the postimmunized sample demonstrated an abundance of intact large nucleated melanoma tumor cells (pink staining). Concurrent staining with the 209-pa HLA tetramer and examination with fluorescent microscopy illustrated the phenotypic presence of g209-pa specific lymphocytes within this lesion (Fig. 3*d*). In contrast, no 209-pa-specific T cells could be identified in the preimmunized specimen (not shown).

Localization of vaccine-induced immune response at the tumor site is not sufficient to cause cancer regression

We next addressed whether peripheral and localized immune response measured in the 209-2M cohort was sufficient to cause tumor regression. Table IV documents individual lesion sizes during 209-2M-based therapy and summarizes the simultaneous analysis of peripheral blood and local tumor reactivity. PBMC reactivity was found in 4 of 9 patients by the IVS assay and in 3 of 9 patients by the direct molecular assay. The magnitude of the direct PBMC reactivity in these patients was low, ranging from 2.2- to 8.1-fold over background. As described in Fig. 2, direct tumor analysis found evidence of immunization in 8 of the 11 lesions. Increases in IFN-γ mRNA levels were apparent in 5 of these lesions (patients 2, 3, 4, 5, and 8) in the absence of measured peripheral blood reactivity by direct molecular assay. Despite even these observed localized responses, none of the 11 lesions showed significant regression in size after vaccination.

Discussion

During the last decade, a major advance in tumor immunology has been the identification of cancer-specific Ags recognized by T cells. These findings have led to strategies to exploit these molecules as cancer vaccines with the purpose of enhancing lymphocyte reactivity. Although clinical tumor regression remains the ultimate goal of immunotherapy, monitoring of in vivo biological effects of immunization may provide clues to the development of more efficacious regimens. Previous in vivo studies have shown that lack of CTL localization at the tumor site correlated with lack of response to adoptive therapy with melanoma-specific CTL (14). However, rarely have direct functional changes within peripheral blood and the tumor microenvironment been used in analyzing the effects of vaccine-based therapy. Most current monitoring protocols have relied on the analysis of in vitro generated PBMC cultures as an indicator of immunization. In this study, we report results of a methodology that allows the direct molecular analysis of immune responses in vivo. We provide evidence demonstrating vaccine-induced lymphocyte reactivity in fresh cells obtained directly from peripheral blood. Induction of gene expression for the activation marker CD69, the cytokines (IFN-γ, GM-CSF, TNF-α, and IL-2), and the proliferation marker CD25 was found directly in postimmunized PBMC after immediate peptide exposure ex vivo. This demonstrated that 209-2M vaccination could result in circulating and functional CTL with activity against the targeted tumor Ag. It is unclear why there was marked quantitative heterogeneity in peripheral blood reactivity among patients receiving identical immunization protocols with the 209-2M peptide. This finding may reflect a biological variable among patients that could play an important role in the optimization of future clinical vaccine regimens.

Our analysis of sequential tumor biopsies during 209-2M-based vaccination (either 209-2M or ES-209-2M; refer to Materials and Methods for details) found that 8 of 11 lesions (73%) demonstrated significant increases in IFN-γ mRNA (paired t test, $p = 0.01$), whereas the control cohorts demonstrated no significant change. Because 209-2M peptide immunization is highly specific in its ability to generate an epitope-specific CTL response, we believe that the changes in the biopsy IFN-γ mRNA levels are indicative of enhanced specific lymphocyte reactivity within the tumor microenvironment. Further, because the tumor-derived IFN-γ mRNA

levels were measured without ex vivo stimulation, we believe that these cytokine mRNA levels are representative of the natural in vivo interaction between vaccine-induced CTL and endogenous tumor Ag. This conclusion is supported by the strong correlation between IFN-γ and gp100 mRNA expression observed in the 209-2M cohort ($r = 0.94$, $p < 0.0001$). Interestingly, five tumor lesions showed treatment-related changes in the absence of detectable peripheral blood reactivity. Local immune changes without detectable systemic precursor reactivity may imply a greater Ag-specific CTL frequency in the local tumor microenvironment than in bulk PBMC, as suggested previously (15). Because of these findings, we believe that sequential analysis of tumors represents the most sensitive and relevant approach to analyzing in vivo effects of cancer vaccines. Further, this molecular methodology would be ideally suited for the assessment of a variety of novel biological agents in clinical trials, if appropriate target tissue is easily accessible.

Unfortunately, despite our evidence of immune reactivity in peripheral blood and at the local tumor site, there was no significant impact of 209-2M therapy on tumor viability and progression. Given that the increase in IFN-γ mRNA in tumors was noted in the absence of significant CD8 and CD4 mRNA increases, we hypothesize that a classically induced response of cellular recruitment and inflammation likely did not occur in these lesions. These results demonstrate the presence but perhaps the limited effectiveness of vaccine-induced T cell response within the target environment. It is possible that the characteristics of Ag presentation exercised by tumors are not optimal to maintain T cells in a state of activation at the tumor site. It has been suggested that tumors induce tolerance by presenting epitope-specific stimulation (signal 1) without costimulation (signal 2) to wandering memory T cells (16). Of interest, our initial evaluation has shown minimal evidence of IL-2 mRNA, a critical growth factor for T cell proliferation, in the tumor microenvironment of vaccine-treated patients (work in progress). Some models predict that in the absence of an ongoing "danger signal," the vaccination response will wane and eventually stop (17). Tumor escape through Ag and/or HLA loss (18) does not fully explain our observed tumor resistance to therapy, given that we had lesions with documented high expression of gp100 and HLA A2 showing no preferential regression. Other possible explanations include the effects of local immunosuppressive factors such as IL-10 and TGF-β (19) and expression of apoptotic signals (20). Recently, it has been reported that effector T cells induced against antigenic tumors could be maintained by prolonged or repetitive vaccination (21). Modifications and adjuvants to immunization schemes, such as exogenous IL-2 and modalities to facilitate CTL help, may provide additional stimuli to heighten the immune reactivity that we have observed into effective antitumor response. Direct serial molecular analysis of gene expression in tumors represents an extremely sensitive and powerful tool to monitor these immunological changes in vivo. This methodology may help further guide the development of future biological therapies.

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EXHIBIT A

1: J Immunol. 2001 Dec 1;167(11):6356-65.

Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity.

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IL-2-dependent activated cells undergo apoptotic death when IL-2 is withdrawn either in vitro or after in vivo cell transfer. To attempt to sustain their survival after IL-2 withdrawal, melanoma-reactive human T lymphocytes were retrovirally transduced with an exogenous human IL-2 gene. Transduced PBMC and cloned CD8+ T cells produced IL-2 and maintained viability after IL-2 withdrawal. Upon restimulation, IL-2 transductants proliferated in the absence of exogenous IL-2 and could be actively grown, and their survival could be maintained without added IL-2 for over 8 wk. PBMCs similarly transduced with a control vector did not produce IL-2 and failed to proliferate in the absence of IL-2. A CD8+ T cell clone, when transduced with an IL-2 gene, manifested the same phenotypes as PBMCs in the absence of exogenous IL-2. Furthermore, an Ab reactive with the alpha-chain of IL-2R complex reduced the viability mediated by IL-2 secretion of the IL-2 transductants. Moreover, transduction of an IL-2 gene did not affect the high degree of recognition and specificity of transductants against melanoma targets. These tumor-reactive IL-2 transductants may be valuable for in vitro studies and for improved adoptive transfer therapies for patients with metastatic melanoma.

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